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Intrauterine position affects motoneuron number and muscle size in a sexually dimorphic neuromuscular system

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Abstract

The intrauterine position occupied by a rodent fetus influences the amount of testosterone to which it is exposed before birth. Animals that are gestated between two male fetuses (2M) are exposed to higher circulating levels of testosterone than are animals positioned between two female fetuses (2F) and there are reliable differences in the reproductive physiology and behavior of 2M and 2F animals when adult. To determine whether intrauterine position modifies development of the central nervous system, we examined the sexually dimorphic spinal nucleus of the bulbocavernosus (SNB) in male and female gerbils from known intrauterine positions. We found that adult 2M female gerbils had 16% more SNB motoneurons than did 2F females. 2M males did not differ from 2F males in SNB motoneuron number, but the bulbocavernosus muscle, which is innervated by SNB motoneurons, was approximately 50% larger in 2M than in 2F males. These data indicate that intrauterine position can influence the morphology of the sexually dimorphic SNB neuromuscular system.

Keywords: Spinal nucleus of the bulbocavernosus; Androgen; Gerbil; Cell death

1. Introduction

The physiology, anatomy and behavior of adult litter-bearing rodents can be influenced by the intrauterine position (IUP) an individual occupies as a fetus. Animals that, as fetuses, are gestated between two males (2M males or 2M females) differ from animals that are gestated between two females (2F males or 2F females) in sexual behavior as well as several measures of reproductive physiology [10,13,15,38,39]. Although such effects have been observed in several rodent species, the impact of IUP on adult phenotype is particularly marked in Mongolian gerbils (*Meriones unguiculatus*), the subject species in the present experiment. As adults, 2M male gerbils scent mark more frequently, mount estrous females with shorter latencies, ejaculate after fewer intromissions, are more successful in impregnating females and are more attractive to estrous females than 2F males [11,13]; anogenital distance, size of ventral scent glands and relative testes weights are all larger in 2M than in 2F male gerbils [11]. The sex of a female gerbil's intrauterine neighbors also influences its later reproductive physiology: 2F female gerbils reach sexual maturity earlier and have higher life-time fecundity than do 2M females [10,12].

Such effects of IUP appear to be mediated by the hormonal milieu to which fetuses are exposed. Fetuses contiguous to males have higher circulating levels of testosterone than do fetuses of the same sex from IUPs adjacent to no males [9,40] and at least some effects of IUP can be prevented by administering steroid-receptor blockers in utero [15]. The precise route by which hormones produced by one animal in a litter reach other individuals is not known and may vary from species to species [25,40].

Since perinatal exposure to testosterone has been shown to mediate the development of several sexually dimorphic neural structures, we reasoned that IUP might have measurable effects on the development of such hormone-dependent neural areas. Structural changes in relevant neural areas might, in turn, underlie some of the well-established differences in reproductive behavior observed in animals from different IUPs. In the present study, we examined the effect of a gerbil's IUP on a sexually dimorphic neural

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system known to be involved in copulation [22,30].

The spinal nucleus of the bulbocavernosus (SNB) of rodents is located in the lower lumbar spinal cord and innervates the bulbocavernosus and levator ani (BC/LA) muscles, which wrap around the base of the penis and the rectum [1,24,33]. These muscles mediate penile reflexes involved in copulation and an intact SNB neuromuscular system is crucial to a male rat's reproductive success [22,30]. Male rats and mice have many more SNB motoneurons than do females [1,41] and the BC/LA muscles are absent or vestigial in females [6,33]. Ulibarri and colleagues have recently identified the SNB of the Mongolian gerbil and found that, as in other mammals, male gerbils possess many more SNB motoneurons than do females [36].

The SNB neuromuscular system is sensitive to circulating androgen levels during early development. SNB motoneuron number and BC/LA muscle mass are permanently masculinized by treating female rats with testosterone during the late prenatal or early postnatal period [4,29]. Although the SNB system has been best studied in rats, motoneurons innervating the perineal muscles exhibit a similar androgen dependence in other mammalian species, including mice, dogs and gerbils [16,19,41]. Because testosterone exposure during the late prenatal period in the gerbil is increased by contiguity to male fetuses [9], we reasoned that intrauterine position might lead to differences in development of the SNB neuromuscular system of Mongolian gerbils. Specifically, we predicted that 2M male and 2M female gerbils would have more SNB motoneurons than their 2F counterparts and that mass of the BC muscle would be greater in 2M than in 2F male gerbils.

2. Materials and methods

In a pilot study we confirmed the position of the SNB nucleus of Mongolian gerbils. A 20% aqueous solution of horseradish peroxidase (3 μl; HRP type VI, Sigma) was injected into the BC and/or LA muscle of three adult males. Following a 24 or 48-h survival period the gerbils were perfused and sections through the lower lumbar spinal cord were stained for HRP reaction product using the method described in Mesulam [26]. In agreement with a previous report [36], we found retrograde-labelled cells lateral, or dorsolateral, to the central canal in a position corresponding to lamina X. This is a very unusual location for spinal motoneurons and the position of the nucleus is depicted in a thionin-stained section in Fig. 1.

2.1. Breeding and determination of intrauterine position

Adult female gerbils (90–100 days of age) were weighed and placed with an adult male. Twenty-four days after a female had been observed to copulate (i.e., 1 day before anticipated vaginal delivery), each female was anesthetized by metofane inhalation, its abdomen opened, the uterus externalized and the fetuses removed one at a time. The gender of each fetus was determined on the basis of its anogenital distance and the position of each fetus in its dam's uterus was recorded. We classified those fetuses

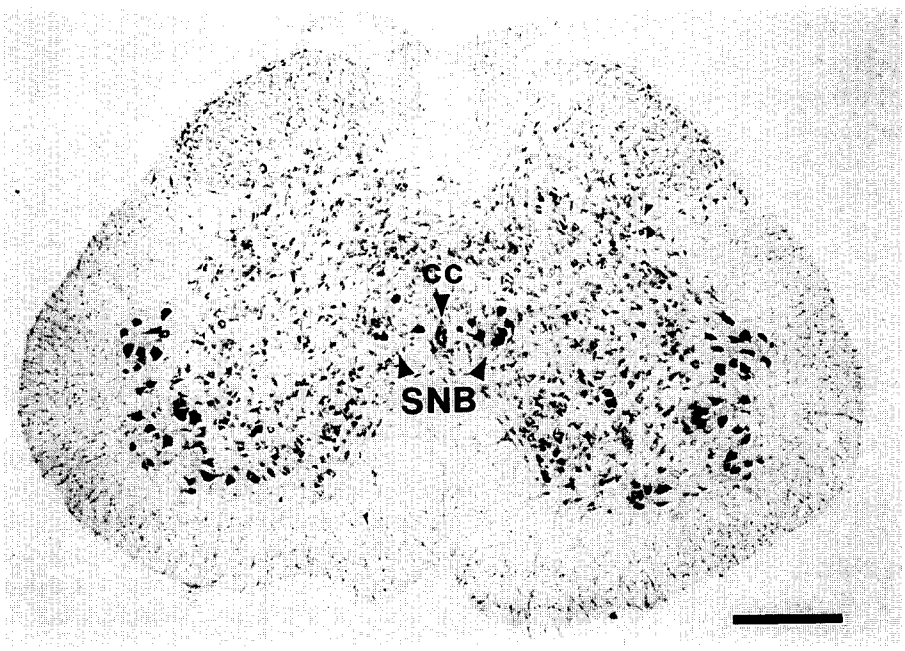

Fig. 1. Photomicrograph of a thionin-stained section through the lower lumbar spinal cord of an adult male gerbil. SNB motoneurons can be seen lateral, and dorsolateral, to the central canal (cc). Scale bar = 300 μm.

located in IUPs between two male fetuses as '2M' and those located in intrauterine positions between two female fetuses as '2F'. Those fetuses in positions between one male and one female and those at either end of a row of fetuses within a uterine horn were not included in the present study. Nineteen male gerbils (6 2M and 13 2F) served as subjects in the first experiment. We later examined the SNB system in 24 females (13 2M and 11 2F). Because the spinal cords of males and females were generated, processed and examined at different times and by different investigators we have analyzed the data for the two sexes separately in the present report. Each of the 43 subjects was derived from a separate litter.

Each infant was toe clipped for permanent identification and cross-fostered to a dam that had vaginally delivered a litter within the past 24 h, using the procedure described by Clark and Galef [10]. Pups were weaned on day 30 postpartum and were left undisturbed in same-sex groups of 4–6 until adulthood.

2.2. Determination of BC/LA muscle mass and SNB motoneuron number

As adults, all animals were tested on several measures of reproductive behavior, as part of a separate study (M. Clark, in preparation). At 6–8 months of age animals were weighed, anesthetized by intraperitoneal injection of sodium pentobarbital and transcardially perfused with saline, followed by buffered formalin (10% formaldehyde w/vol). The BC/LA muscle complex was dissected out of the males, trimmed and weighed by an individual blind to the IUP of each animal. The BC/LA complex was not clearly discernable in 2M or 2F females by gross dissection and therefore was not dissected out or weighed.

Spinal cords were post-fixed in buffered formalin for at least 3 weeks. The lumbosacral portion of the spinal cord was cryoprotected by soaking in a 10% sucrose solution overnight and then frozen sectioned in the coronal plane at a thickness of 40 μm. Alternate sections were mounted on separate slides and stained with thionin. All counts and measurements were made on slides that were coded to conceal the animal's IUP. Bilateral counts of SNB motoneurons were made on alternate sections for males and on every section for females. Motoneurons were recognized by their position, large size and darkly staining somata. Only those SNB cells in which the nucleus was visible were included in the counts.

The soma and nucleus of at least 20 SNB motoneurons from each animal were drawn by camera lucida and cell size was determined with the aid of a digitizing pad linked to a microcomputer. Sections chosen for tracing were equally spaced throughout the rostro-caudal extent of the SNB nucleus and all possible SNB motoneurons were traced from each selected section to avoid experimenter bias. Raw motoneuron counts were then corrected for sampling ratio and for split nuclei using the method described in Konigsmark [23], as the size and shape of motoneuronal nuclei closely conform to the assumptions of this counting method.

Student's *t*-tests for independent samples were employed for within-sex comparisons. All probabilities reported are two-tailed and means are reported ± one standard error of the mean (S.E.M.).

3. Results

Males had more than 5 times as many SNB motoneurons than did females (222.5 ± 6.4 vs. 40.8 ± 1.5; Fig. 2 and Fig. 3) and our cell counts conform closely to those reported previously for male and female gerbils [36].

Intrauterine position did not affect SNB cell number in males (Fig. 2A) and there was no significant difference in the size of SNB cell somata or nuclei between 2M and 2F

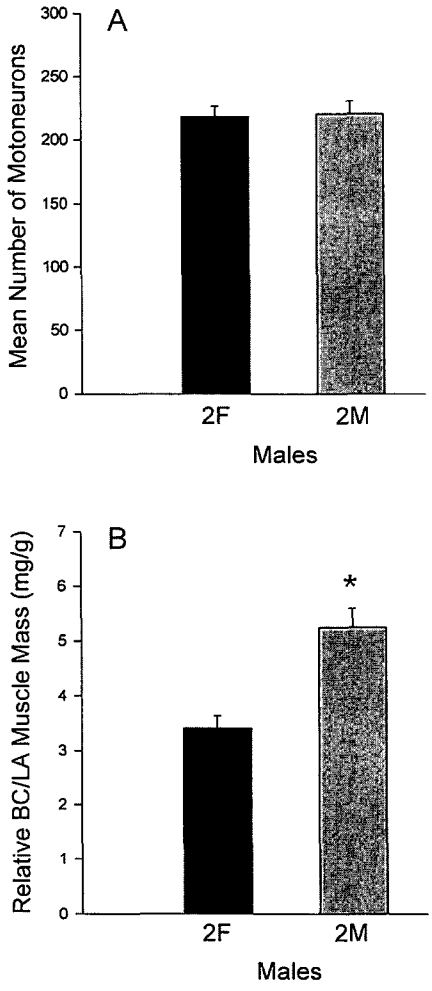

Fig. 2. A: the mean (±S.E.M.) number of motoneurons in the SNB region of 2F and 2M males gerbils. B: mass of the BC/LA muscle complex, expressed relative to body mass, in adult male gerbils from 2F and 2M intrauterine positions. 2M males had significantly larger BC/LA muscles than did 2F males ($P < 0.001$).

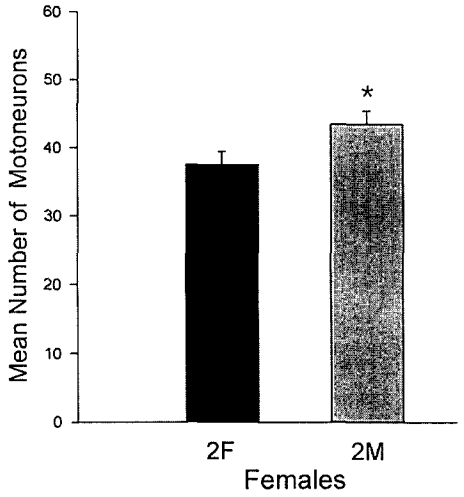

Fig. 3. The mean number of motoneurons in the SNB nucleus of adult females from known intrauterine positions. 2M females had significantly more SNB cells than did 2F females ($P < 0.05$).

males (not shown). However, the BC/LA muscle complex was larger in 2M than in 2F males. This difference in the size of the BC/LA muscle complex was significant whether expressed as absolute BC/LA mass (45% larger in 2M males; $t = 3.45$, $P < 0.005$), or BC/LA mass relative to body mass (53% larger in 2M males; Fig. 2B; $t = 4.52$, $P < 0.001$). Body mass did not differ significantly between the two male groups, although there was a tendency for 2M males to be lighter than 2F males (2M: 87.0 ± 1.3 g, 2F: 98.5 ± 4.4 g; $t = 1.71$, $P > 0.05$).

There was also a significant effect of IUP on SNB motoneuron number in female gerbils: 2M females possessed about 16% more cells in the SNB than did 2F females (Fig. 3). This difference in SNB motoneuron number was observed both for raw motoneuron counts and in counts corrected for cell size ($t = 2.25$ (raw counts) and $t = 2.13$ (corrected counts), $P < 0.05$ in each case). The size of SNB motoneuronal somata and nuclei was not significantly different in 2M and 2F females and body mass did not differ between the two female groups (2M: 86.4 ± 5.1 g, 2F: 84.7 ± 6.0 g; $t = 0.22$, $P > 0.50$). The BC muscle could not be discerned in females by gross dissection, as described above.

4. Discussion

Development of the SNB neuromuscular system is androgen dependent. In rats, SNB motoneurons and their target muscles initially form in both sexes. However, because development of this system is critically dependent upon androgen, most SNB motoneurons of females die during the perinatal period [4,6,29]. SNB cell number and BC/LA size are partially masculinized in females by either late prenatal or early postnatal testosterone treatments [4]. When female rats are exposed to high levels of testosterone throughout both pre- and early post-natal development, the SNB nucleus is completely masculinized and SNB cell number is not distinguishable from that of males [29]. That androgens, and not estrogenic metabolites of testosterone, are required for these hormonal effects is demonstrated by the observations that: (1) perinatal estrogen administration does not increase SNB motoneuron number [5] and (2) mutant male rats lacking functional androgen receptors develop a completely feminine SNB [2,32].

Intrauterine position influenced SNB cell number in female gerbils, such that 2M females possessed more SNB motoneurons than did their 2F counterparts. However, this increase in SNB cell number was relatively modest and SNB cell number of 2M females was still substantially lower than that observed in males. If findings in rats can be extrapolated to gerbils, it is likely that increased testosterone levels experienced prenatally by 2M female gerbils rescued a portion of the SNB motoneurons that normally die in 2F females, but that complete masculinization of the gerbil SNB would require higher prenatal testosterone levels and/or more protracted exposure to elevated testosterone than is normally experienced by 2M females. Indeed, preliminary findings indicate that, as in rats, both pre- and postnatal testosterone contribute to SNB cell survival in gerbils [16].

Intrauterine position does not appear to influence SNB motoneuron number in male gerbils, as 2M and 2F males did not differ in this measure in the present study. A near complete blockade of androgen stimulation by perinatal anti-androgen treatments significantly suppresses SNB motoneuron number in male rats [3]. However, more subtle decreases in androgen stimulation have only minor effects on development of the male SNB. For example, prenatal stress, which lowers but does not abolish the prenatal testosterone surge in male fetuses, results in a 3% decrease in SNB cell number in male rats [21]. In addition, SNB cell number is not altered by treating male rats with supplemental exogenous testosterone during late prenatal and early postnatal development [31]. Taken together, these observations suggest that there is normally more than enough testosterone circulating in perinatal male rats to maximally masculinize SNB motoneuron number and that moderate alterations in testosterone levels, such as the those induced by prenatal stress, do not markedly alter SNB cell survival. By analogy to the findings in rats, the differences in androgen stimulation experienced by 2M and 2F male gerbils may not be great enough to produce significant effects on SNB motoneuron number. Because the number of 2M males in the present study was not large, however, we cannot exclude the possibility that IUP contributes to subtle changes in SNB cell number in males too small to be detected here.

Mass of the BC/LA muscle complex was significantly greater in 2M than in 2F males. There are at least two

possible explanations for this difference. Prenatal testosterone permanently increases BC/LA muscle size in rats, by increasing both muscle fiber number and muscle fiber size [6,34,35]. Similarly, the elevated levels of testosterone experienced by 2M male gerbils prenatally [9] may have resulted in enhanced development of the BC/LA. An effect of IUP on development of the perineal muscles, in the absence of a similar effect on SNB motoneuron number, suggests that BC/LA muscle size is more sensitive to small changes in hormone availability than is SNB cell number. Alternatively, the larger BC/LA sizes in 2M males may result, not from developmental hormone effects, but from differences in circulating levels of testosterone in adulthood. Size of the BC/LA muscle complex is highly sensitive to circulating androgen levels in adult rats [37,42] and 2M male gerbils have larger relative testes weights and higher levels of serum testosterone as adults than do 2F males [11,14]. Further, 2M male gerbils may be more responsive to a constant dose of testosterone in adulthood than are their 2F counterparts, an effect that is likely mediated by increased exposure to androgen in utero [7].

Since SNB motoneuron number was elevated in 2M females, one might expect that the BC/LA muscles would be similarly masculinized in these animals. In rats, androgen effects on SNB cell survival appear to be mediated by hormone action at the BC/LA target muscles [17,18]. While we could not discern the BC/LA in 2M or 2F female gerbils by gross dissection, it is possible that a microscopic analysis of sections through the perineum would reveal small changes in the sizes of these muscles. Size changes might also be measurable if females were treated with testosterone before being killed to maximally stimulate any muscle fibers that are present. It is also possible, of course, that testosterone stimulates SNB cell survival not by mediating alterations in muscle size, per se, but by stimulating the production and release of neurotrophic molecules from the BC/LA (cf. [20]).

An additional indirect mechanism whereby IUP may influence the SNB is via effects on maternal behavior. It is well established that both rat and gerbil mothers spend more time licking the anogenital region of male than of female offspring [8,28]. When the level of maternal licking is experimentally reduced, a significant, 11%, decrease in SNB motoneuron number results in rats [27]. Since gerbil dams spend more time licking the anogenital region of neonates from 2M intrauterine positions of each sex [8], alterations in perineal stimulation could account for masculinization of SNB cell number or BC/LA size seen in 2M animals in the present study. Regardless of mediating mechanisms, the influence of IUP has now been shown to include effects on the nervous system. At least some within-sex individual variation in the SNB neuromuscular system and perhaps in other sexually dimorphic neural systems as well, may be attributed to the IUP occupied by an individual during fetal development.

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